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PRINCIPAL INVESTIGATOR: Maria Jasin, Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Institute for Cancer Research
New York, New York 10021

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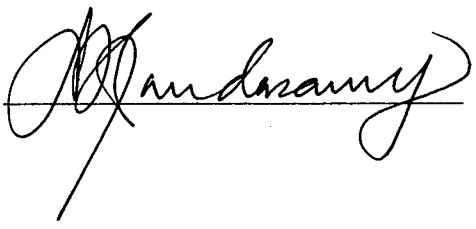

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Maria Jasin, PhD
Cell Biology Program

December 15, 1999

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Sincerely,

Maria Jasin, Ph.D.
Associate Professor

Memorial Sloan-Kettering Cancer Center
1275 York Avenue, New York, New York 10021
Telephone 212.639.7438 • FAX 212.717.3317
E-mail: m-jasin@ski.mskcc.org

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Maria Jasin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Sloan-Kettering Institute for Cancer Research
New York, New York 10021

E-MAIL:
m-jasin@ski.mskcc.org

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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this research is to determine a role for BRCA1 and BRCA2 in the recombinational repair of DNA double-strand breaks. The inability of a cell to precisely repair chromosomal breaks may lead to genetic instability and consequently promote carcinogenesis. Products of the BRCA1 and BRCA2 genes have been shown to interact with Rad51, a component of homologous repair pathways known to precisely repair breaks. In this annual report we describe a homologous gene targeting defect in Brca1-deficient ES cells. We also measured gene conversion within a substrate containing GFP gene repeats, one of which is mutated by the recognition site of the rare-cutting I-*Sce* I endonuclease. Following expression of I-*Sce* I, repair of an induced break can be monitored by measuring green fluorescence within the cell. We have found that repair of a chromosomal break by gene conversion is diminished in the Brca1-deficient ES cells. Additionally, we have analyzed gene targeting in ES cells that contain a truncated Brca2 gene. These results directly demonstrate that cells deficient in Brca1 have reduced homologous repair of an induced chromosomal double-strand break. This defect may contribute to the propensity to develop early-onset breast and ovarian cancers by increasing genetic instability.

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Maria Jaen 12/17/99

PI - Signature Date

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INTRODUCTION

In mammals cells, homologous recombination is expected to maintain genomic integrity through the precise repair of lesions such as chromosomal double-strand breaks (DSBs). For precise repair, the sister chromatid would be expected to be the preferred repair template, as sister-chromatids are identical to each other (1). Nevertheless, repair of chromosomal DSBs using other homologous templates, such as alleles or repetitive elements, may be deleterious or catastrophic, since it can result in loss of heterozygosity or translocations, respectively (2, 3). Thus, disruption of genes involved in DSB repair processes may be predicted to disturb the balance of various repair pathways that have evolved to guard the genome from perturbation. An essential component of homologous recombination pathways is the strand-exchange protein RAD51. The products of both hereditary breast cancer genes, BRCA1 and BRCA2, co-localize with RAD51 to nuclear foci following DNA damage in mitotic cells and at forming synaptonemal complexes in meiotic cells (4, 5). BRCA1-deficient human cells and Brca1-deficient murine are sensitive to DNA damaging agents which create DSBs, implicating these proteins in DSB repair (6). Our goal was to determine if mutation of BRCA1 or BRCA2 affects homologous recombination.

BODY

Statement of work:

Task: To analyze gene targeting and the recombinational repair of induced DSBs in BRCA1-deficient ES cells.

- Quantitate gene targeting efficiencies in BRCA1-deficient and parental ES cells using pimhyg targeting constructs; analyze the fidelity of the gene targeting event by Southern and PCR analysis
- Create additional recombination substrates cloned within the pimhyg targeting construct to test the role of BRCA1 in the recombinational repair of additional I-*Sce* I repair templates

The *Brca1*$^{-/-}$ 236.44 and control *Brca1*$^{+/-}$ 310.7 cell lines (7) were tested for gene targeting proficiency at the *pim1* locus on chromosome 17, using a previously published pimhyg targeting design (8, 9). The previously published design was modified to create an additional recombination substrate cloned within the pimhyg targeting vector that will be described in detail below. This additional recombination substrate is for the analysis of DSB-promoted gene

conversion, creating for the first time a marker assay for gene conversion in Brca1-deficient cells.

The pimhyg gene targeting vectors contain a promoterless hygromycin resistance (hyg^R) gene cloned in frame with *pim1* coding sequences. The *hyg* gene is expressed when the vector correctly gene targets at the *pim1* locus or when a fortuitous nonhomologous integration occurs adjacent to promoter sequences. The pimhyg targeting vectors (p59DRGFP4 and p59DRGFP6) were electroporated into both cell lines and hyg^Rcolonies were selected. Southern analysis demonstrated that most of the hyg^R $Brca1^{-/-}$ clones were derived from random integration of the targeting vectors, with only 15% of clones derived from gene targeting (Table 1). By contrast, efficient gene targeting was observed in the $Brca1^{+/-}$ and $Brca1^{+/+}$ cell lines, with greater than 90% of the clones correctly targeted.

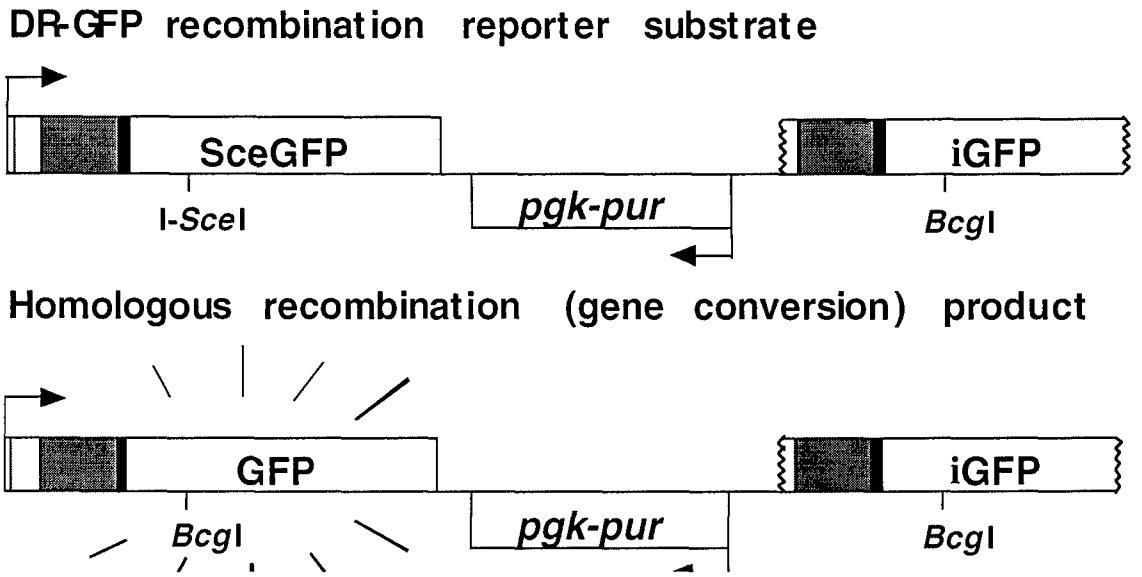
Table 1

Cell line	pim1 Targeting Efficiency	
	p59DRGFP4	p59DRGFP6
ES TG2A ($Brca1^{+/+}$)	Not Done	88/90 = 97.8%
310.7 ($Brca1^{+/-}$)	63/72 = 87.5%	86/93 = 92.5%
236.44 ($Brca1^{-/-}$)	5/32 = 15.6% (5.6X decrease)	8/54 = 14.8% (6.3X decrease)
$Brca2^{lex1/lex2}$	25/42 = 59.5%	39/79 = 49.4%

We next investigated the Brca1-deficient and control ES cell lines for the repair of chromosomal DSBs by intrachromosomal gene conversion. During the process of gene targeting, a gene conversion substrate called DR-GFP (10) was integrated at the *pim1* locus, since this substrate had been previously cloned into the pimhyg targeting vector. DR-GFP is composed of two differentially mutated green fluorescent protein (GFP) genes (Figure 1). GFP serves as marker gene since a GFP+ gene indicates that a gene conversion event occurred. The two GFP genes are oriented as direct repeats and separated by a drug selection gene for puromycin resistance (10). The SceGFP gene is mutated to contain the recognition site for I-*Sce* I, a rare-cutting endonuclease, so that DSBs can be induced in the substrate *in vivo*. The I-*Sce* I site was introduced in the GFP gene at a *Bcg* I restriction site by substituting 11 base pairs (bp)

of wild-type GFP sequences with those of the I-*Sce* I site. These substituted bp also supply two in-frame stop-codons, which terminate translation and inactivate the protein. Downstream of the SceGFP gene is a 0.8 kb internal GFP fragment termed iGFP, and the two homologous mutated GFP genes are separated by 3.7 kb.

Figure 1 Taken from Pierce et al. (1999).



Gene conversion initiated by a DSB at the I-*Sce* I site restores an intact GFP gene whose expression is detected by cellular fluorescence. The two gene targeting vectors, p59DRGFP4 and p59DRGFP6, each contain the DR-GFP gene conversion substrate, although it is in opposite orientations in the two vectors. Two gene targeted cell clones were analyzed for each cell line and for each orientation of the DR-GFP substrate. Cells were electroporated with an I-*Sce* I expression vector or control DNAs and then analyzed for fluorescence on a flow cytometer. Results are shown in Table 2.

The *Brca1*$^{+/-}$ cell clones had average gene conversion frequencies of 1.17 and 2.05% for the two different orientations, whereas the *Brca1*$^{-/-}$ cell clones had average frequencies of 0.15 and 0.32%, respectively. Thus, DSB-promoted gene conversion is decreased with *Brca1* mutation. This is consistent with our initial hypothesis that BRCA1 plays a key role in recombination repair of chromosomal breaks. Gene conversion is a very precise form of DSB repair and is not mutagenic (i.e., is genetically silent) when it occurs between sister-chromatids.

Our previously published work has demonstrated a relationship between direct repeat recombination, as in our gene conversion substrate DR-GFP, and sister-chromatid recombination. Therefore, *BRCA1* mutation appears to alter DSB repair.

Table 2

		% GFP positive cells	
Cell clone	DNA*:Neg	I-Sce I	NZE
310.7 DRGFP6 C6	0.00	1.82	85.6
310.7 DRGFP6 H2	0.00	0.51	62.9
		Avg = 1.17	
236.44 DRGFP6 B1	0.01	0.14	66.4
236.44 DRGFP6 F6	0.01	0.16	ND
		Avg = 0.15 (7.8X dec)	
310.7 DRGFP4 B6	0.00	2.02	80.5
310.7 DRGFP4 B12	0.00	2.07	89.7
		Avg = 2.05	
236.44 DRGFP4 B2	0.00	0.35	70.6
236.44 DRGFP4 H5	0.00	0.28	88.3
		Avg = 0.32 (6.4X dec)	

*DNA: Neg, mock DNA; I-Sce I, the pCBASce I-*Sce* I expression vector;
NZE, a GFP expression vector.

Task: To specifically disrupt the BRCA2 interaction with Rad51 during induced I-*Sce* I chromosomal cleavage for the analysis of LOH and other types of DSB repair

- create dominant-negative expression plasmids for the wild-type (and, for a control, mutant) open reading frames of BRCA2 for functional inhibition of BRCA2/Rad51 protein interactions

We are currently creating the dominant-negative expression plasmid. In addition to disrupting the BRCA2-Rad51 interaction with a dominant negative expression plasmid, we have recently obtained an ES cell line with *Brca2* alleles disrupted by gene targeting (11). The disruption is in a presumed interacting domain of mouse Brca2 with Rad51. This recent development presents a very precise system for analyzing the effect of *Brca2* disruption, as every cell will have the protein disrupted.

We have begun our analysis of DSB repair by introducing the DR-GFP substrate into the *Brca2* disrupted cell line, $Brca2^{lex1/lex2}$. As can be seen in Table 1, we have obtained cell lines

containing the GFP substrate in both orientations. We have not, as yet, performed the DSB repair assay. Thus, we cannot reach any conclusions. The gene targeting frequencies, however, suggest that recombination may be slightly impaired in the $Brca2^{lex1/lex2}$ cell line.

KEY RESEARCH ACCOMPLISHMENTS

- Created a marker gene conversion recombination substrate cloned within the pimhyg targeting construct to test the role of Brca1 in the recombinational repair of an I-*Sce* I-induced chromosomal DSB
- Quantitated gene targeting efficiencies in Brca1-deficient and parental ES cells using the pimhyg targeting constructs containing the marker gene conversion substrate
- Quantitated DSB-induced gene conversion in Brca1-deficient and parental ES cells using pimhyg targeting constructs
- Quantitated gene targeting efficiencies in Brca2-deficient and parental ES cells using the pimhyg targeting constructs containing the gene conversion substrate

REPORTABLE OUTCOMES

- Development of ES cell lines deficient in Brca1 and Brca2 which contain gene conversion recombination reporter marker substrates at the *pim1* locus

CONCLUSIONS

Our results support the hypothesis that BRCA1 and possibly BRCA2 play a role in recombinational repair and therefore have a caretaker role in the cell, guarding against tumorigenesis.

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